64-9

Annual Report to the Commission on Immunization

of the

Armed Forces Epidemiological Board

(a) Project Title

Studies on the mode of action of Diphtheria Toxin

(b) Principal Investigator:

A. M. Pappenheimer, Jr.
Professor of Biology

(c) Research Associate:

Pauline Miller, R. John Collier

(d) Contract Number:

DA-49-007-MD-994

(e) Institution:

Biological Laboratories
Harvard University
Cambridge 38, Massachusetts

(f) Report Period:

March 1, 1963 - February 28, 1964

NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.

ABSTRACT

1. Annual report for period March 1, 1963 - February 28, 1964

2. Principal Investigator:

A. M. Pappenheimer, Jr.
Professor of Biology
Harvard University
Cambridge 38, Massachusetts

3. Contract Number:

DA-49-007-MD-994

I. Mode of action of diphtheria toxin: Using tritium-labelled toxin, it has been shown that HeLa cells treated with a saturating dose take up less than 2% (0.005ug/ml) of the added toxin within a 5-hour period. Diphtheria toxin exerts its action on cells by inhibition of protein synthesis. Low concentrations of toxin block the incorporation of C14-amino acids into protein in extracts of HeLa cells and of rabbit reticulocytes. Action of the toxin requires the presence of a specific cofactor which has been identified as nicotinamide adenine dinucleotide (NAD). It has been shown that the toxin interferes with a step involving the transfer of aminoacids from aminoacyl-sRNA to the growing polypeptide chain.

II. Studies on diphtherial phages: A method for phage assay has been developed using an anti-head protein serum gammaglobulin fraction, trace labelled with I^{131}. Bacteria to which 4 or more phage particles have been absorbed are specifically agglutinated by the serum. The agglutinate is collected on Millipore filters and counted for I^{131}. The method has proved useful in studying the defective non-plaque forming phages released by strains P(βd) and PWB. Following induction of P(βd) by UV light, the burst size is the same as for the normal lysogenic strain, P(β). The βd phage absorbs normally to the sensitive C7(-) strain and using P^{32}-labelled βd, it has been found that its DNA is injected normally. As determined by the I^{131} method, the classic PWB strain releases less than 2% as much absorbable phage following UV induction as do P(β) and P(βd) under similar conditions.

MODE OF ACTION OF DIPHTHERIA TOXIN

It was shown by Strauss and Hendee (1), that following addition of a "saturating dose" (0.5µg/ml) of purified diphtheria toxin to a growing culture of HeLa cells, growth and protein synthesis continue at their normal rate for 1.5-2 hours and then cease abruptly. Very much lower concentrations of toxin will kill mammalian cells if left on contact with them for a prolonged length of time. In fact it can be calculated from the experiments of Lennox and Kaplan (2) and of Gabliks and Solotorofsky (3), that toxin at a concentration of less than 10^{-13}M equivalent to only 200-400 molecules per cell, is lethal for certain strains of mammalian cells within 3-4 days. How much toxin is actually taken up by the cells when toxin is added to a suspension in saturating concentrations? In an attempt to answer this question, we have prepared highly purified tritium-labelled toxin and attempted to measure its uptake by cultures of the S3 strain of HeLa cells.

Tritiated toxin was prepared by growing the SM-1 variant of the PW8 strain in Yoneda's medium (4) containing uniformly labelled H^3-l-methionine. The H^3-toxin was purified by ammonium sulfate fractionation, followed by passage through Sephadex G-25 and finally by fractionation on a DEAE column. The final product contained 2.5µg protein and 60mld per Lf and 160 counts per min per µg. In a typical experiment, three culture flasks were inoculated, each with 10^5 HeLa (S3) cells per ml. To flasks A and B were added ca 7×10^4 cpm/ml C14-phenylalanine; to flasks B and C were added 1.7µg/ml tritiated toxin. At hourly intervals one ml samples were withdrawn and the cells collected on Millipore filters. Cells from flasks A and B were counted for C14; those from flask C for tritium. The C14 counts showed that phenylalanine uptake in the flasks containing toxin began to slow down and deviate from the control after 2 hours and had come to a standstill within 5 hours. Table 1 shows the results of the parallel experiment (flask C) to which labelled toxin but no C14-phenylalanine was added. The radioactivity recovered with the cells averaged only 2.5% of the total counts added, did not change significantly with time over the 5 hour period and was uneffected by washing. In fact the counts were not appreciably greater than in zero time controls in which toxin was rapidly passed through Millipore filters on which 10^6 HeLa cells had previously been collected. At the end of the 5 hour period, up to 90% of the labelled toxin could be recovered from the filtrates by co-precipitation with added unlabelled toxin and an equivalent amount of anti-toxin.

It seems clear from the results of Lennox and Kaplan and of Gabliks and Solotorofsky referred to above, that only a small fraction of a saturating dose would need to pass the cell membrane in order to kill a cell. At the extremely low concentrations that they used (ca 10^{-13}M or 200-400 molecules per cell) diffusion may be a limiting factor in determining the length of the latent period. If the toxic protein is taken up by pinocytosis, then the cells must imbibe large amounts of culture fluid, since the total cell volume in their experiments was less than 0.2%. The experiments with labelled toxin have failed to tell us just how much toxin is fixed by sensitive cells.

Nevertheless, they do demonstrate that the amount of toxin which is fixed or passes through the cell membrane is very small indeed and may be only a few molecules per cell, even in the presence of high toxin concentrations. This fact is of significance in considering the mode of action of diphtheria toxin, since it seems to preclude the likelihood that cell damage is the result of one to one interaction of the toxin molecule with a stable cell component such as an enzyme. It suggests, rather, that the toxin may act catalytically to activate a degradative enzyme or to inactivate a vital enzyme or other cell catalyst.

Table 1

Uptake of H^3-methionine-labelled diphtheria toxin by HeLa cells (S3) in spinner culture

Time (hours)	Counts per minute per 10^6 cells[a]			
	Unwashed	Washed with Medium alone	Washed with medium and 5% TCA	
			(a)	(b)
0	6.8[b]	-	-	-
1	10.8	9.3	8.7	8.0
2	9.1	8.7	8.3	4.5
3	6.5	10.5	7.2	9.2
4	7.3	6.8	11.9	5.1
5[c]	5.8	9.8	8.4	3.0
	(8.0)	(9.0)	(8.7)	(6.0)

[a] One ml samples collected on Millipore filters and counted. 10^6 HeLa cells per ml and 1.7µg (275 cmm) per ml highly purified H^3-methionine-labelled toxin.

[b] At zero time 10^6 normal HeLa cells collected on filter. Then 1 ml containing 1.7µg toxin rapidly filtered through.

[c] After 5 hours, 50-90% of the labelled toxin was recovered from the supernates by specific coprecipitation with added unlabelled toxin and antitoxin.

[d] Figures in parentheses indicate averages over the entire period.

Effect of diphtheria toxin on amino acid incorporation in vitro: Last year we reported preliminary experiments by R. J. Collier which showed that in the presence of only 0.5µg/ml purified toxin, amino acid incorporation into polypeptides by cell-free extracts from HeLa cells was inhibited 50%. The inhibition was shown to be specific for toxin itself, even though antitoxin failed to neutralize the effect completely. Indeed, washed specific toxin-antitoxin flocculates are still capable of inhibiting, almost completely, the incorporation of amino acids by the cell-free extract.

These experiments have now been confirmed and extended. It has been shown that toxin interferes in some way with the transfer of amino acids from aminoacyl-sRNA to the growing polypeptide chain. In order for toxin to act, a specific cofactor is required which has recently been identified as nicotinamide adenine dinucleotide (NAD).

Inhibition of polyphenylalanine synthesis: It seemed possible that toxin might be acting to inhibit selectively the incorporation of only one of the 20 aminoacids into protein. It therefore seemed worthwhile to test the effect of toxin on the incorporation of C14-phenylalanine in the presence of the synthetic messenger RNA, polyuridylic acid, which codes for the formation of polyphenylalanine. Table 2 shows that poly-U-stimulated incorporation of phenylalanine by HeLa cell extracts is indeed blocked by low concentrations of toxin. It would thus appear that if toxin inhibits the incorporation of only one of the amino acids, then that amino acid must be phenylalanine. This possibility seems unlikely.

Table 2

Effect of diphtheria toxin on poly-U-stimulated incorporation of C14-phenylalanine in HeLa cell extracts*

Additions	C-14-phenylalanine incorporation cpm/sample
None	275
Poly-U, 50µg	1402
Poly-U, 50µg toxin, 15µg	206

* The complete system contained 1 µmole ATP, 0.25 µmole GTP 10 µmole creatine phosphate, 120 µmole creatine kinase, 50 µl amino acid mixture, 0.05 µmole (0.6 µc) C14-phenylalanine, 20 µmole GSH, 50 µmole KCl, 5 µmole $MgCl_2$, H_2O, 50 µmole Tris pH 7.5, and 0.4ml HeLa extract to a final volume of 1.4ml.

Effect of toxin on hemoglobin synthesis: Because of the technical difficulties of growing large numbers of HeLa cells, even in spinner cultures, we decided to test the effect of toxin on amino acid incorporation into hemoglobin by a partially purified cell-free system prepared from rabbit reticulocytes. This system

has been extensively studied by Allen and Schweet (5), Dintzes (6) Warner et al (7) and others. Reticulocytes were collected from phenylhydrazine-poisoned rabbits and were extracted and fractionated accordint to Allen and Schweet (5). Ribosomes were separated by high speed centrigugation and then activating and transfer enzymes together with ribonucleic acids were precipitated from the supernate with ammonium sulfate. The complete system when supplemented with Mg^{++}, K^{+}, GTP, amino acids, ATP and ATP-generating system was highly active in incorporating C14-amino acids into TCA precipitable material. Much to our surprise, however, the system proved to be completely insensitive to the action of diphtheria toxin. (Table 3)

Table 3

Incorporation of C14-phenylalanine by cell-free system from rabbit reticulocytes*

	C14-phenylalanine incorporated cpm/sample
Complete system	1040
No ATP, ATP-generating system or GTP	7
No ribosomes	0
No AS70 fraction*	128
No sRNA	628
RNAase, 50µg	4
puromycin, 100µg	49
Diphtheria toxin, 15µg	1046

* Complete system same as in Table 2 except that instead of HeLa extract there was added: 100µg reticulocyte sRNA, 300µg ribosomes and 5mg AS70 fraction (proteins precipitated between 40 and 70% saturation with ammonium sulfate from reticulocyte supernate).

Our first reaction to this finding was that the insensitivity to toxin might be related to the fact that the ribosomes in reticulocytes are not bound to the endoplasmic reticulum as is the case in HeLa cells. This hypothesis was tested by examining the sensitivity to toxin of reticulocyte ribosomes plus the soluble factors from HeLa extracts and of HeLa microsomes plus reticulocyte enzymes and RNA. Table 4 shows clearly that the state of binding of ribosomes does not determine sensitivity to toxin. It is apparent from the

table that only those reaction mixtures containing the HeLa supernate, either alone or in combination with reticulocyte soluble factors, are inhibited by the toxin. This suggests that there is some factor contained in the HeLa supernate, but not present in the reticulocyte soluble fraction, which is necessary for action of the toxin.

Table 4

Effect of toxin on C14-leucine incorporation in cell-free extracts

Source of Ribosomes	Source of soluble factors	C14-leucine incorporated (cpm/sample)*		Inhibition %
		Control	Toxin (15µg/ml)	
reticulocyte	reticulocyte	1414	1418	0
reticulocyte	HeLa	1776	355	80
HeLa	reticulocyte	292	311	0
reticulocyte	reticulocyte + HeLa	2166	672	69

* 0.6µc C14-leucine added to each reaction mixture

Identification of cofactor required for toxic action: It soon became evident that the cofactor present in the HeLa supernate was a small molecule. After dialysis or passage through Sephadex G25, the HeLa supernate retained its full amino acid incorporating activity when supplemented with the microsomal fraction. However, dialysed or gel-filtered supernates were completely insensitive to inhibition by toxin. Sensitivity could be restored by addition of a boiled extract of HeLa cells from which coagulated protein had been removed. Addition of boiled HeLa extract also rendered the reticulocyte system sensitive to inhibition by toxin.

Before attempting to fractionate boiled extracts, various known cofactors were tested for their ability to restore sensitivity of the dialysed system to toxin. The factors tested included flavin nucleotides, pyridine nucleotides (NAD and NADH), pyridoxal phosphate and a yeast concentrate. Only NAD, NADH and yeast extract (containing NAD) restored the sensitivity of the dialysed HeLa system and of the reticulocyte system to toxin. Table 5 shows that in the presence of only 0.5µg NAD per ml, there was about 50% inhibition of incorporation of C14-leucine in the reticulocyte system by 6Lf/ml toxin. The table shows that 50-100 times as much NADP were required for 50% inhibition by toxin in the same system (there were probably traces of NAD in the NADP preparation). It thus seems virtually certain that the dialysable cofactor in HeLa supernates is NAD.

Table 5

Effect of NAD and NADP on inhibition by toxin of incorporation of C14-leucine by reticulocyte system.

Toxin added µg	Cofactor added µg	C14-leucine incorporated* (cpm/sample) NAD	NADP
0	0	549	
15	0	543	
0	50	539	525
15	0.1	489	539
15	0.5	371	534
15	2.5	213	489
15	50	142	333

*0.6µc C14-leucine to each reaction mixture

Effect of toxin on aminoacyl-sRNA formation: Protein synthesis may be considered to take place in two steps: 1. a reaction of amino acids and ATP with activating enzymes and sRNA's specific for each individual amino acid to form aminoacyl-sRNA's and 2. transfer of amino acids from aminoacyl-sRNA to the growing polypeptide chain on ribosomes held together by messenger mRNA. Reaction 2 is catalysed by one or more transfer enzymes. The effect of toxin on step 1 was tested by following, at 15C, the uptake of C14-algal protein hydrolysate by reticulocyte sRNA with time in the presence of NAD (50µg/ml) and activating enzymes from reticulocytes. Toxin proved to be completely without effect on aminoacyl-sRNA formation.

Effect of toxin on the transfer reaction: Purified aminoacyl-sRNA was prepared from rat liver sRNA and C14-phenylalanine or C14-algal protein hydrolysate in the presence of activating enzymes from rabbit reticulocytes. The transfer of the labelled amino acids from the purified C14-aminoacyl-sRNA to hot TCA insoluble polypeptides was carried out by incubation with the complete incorporating system containing AS-70 proteins and ribosomes from reticulocytes and 50µg/ml NAD. Table 6 shows that polypeptide formation was indeed inhibited 40-65% by low concentrations of toxin.

Table 6

Inhibition of transfer of C-14 amino acids

from purified C14-aminoacyl-sRNA to protein by toxin

Aminoacyl-sRNA	Supplements or deletions from complete system	C-14 amino acid incorporated (cpm per sample)		Inhibition %
		Control	Toxin (15ug/ml)	
C14-algal protein hydrolysate	none	3200	3180	0.1
"	50ug NAD	3230	1980	39
"	50ug NAD 1ug each of 18 amino acids	3580	1280	64
"	no ribosomes	55	-	-
C14-phenylalanine	none	825	815	1
"	50ug NAD	784	425	46
"	50ug poly-U	1318	1119	15
"	50ug NAD 50ug poly-U	1229	465	63

Conclusions: Low concentrations of highly purified diphtheria toxin inhibit incorporation of amino acids into protein by extracts of HeLa cells and by rabbit reticulocytes. NAD is a required co-factor for this inhibition. The site of action of the toxin appears to be on some component involved in the transfer of amino acids from sRNA to the growing polypeptide chain. It is not known, as yet, whether toxin is itself an NAD requiring enzyme that degrades an essential component in the reaction or whether toxin activates a degradative enzyme that requires NAD as a cofactor.

II

STUDIES ON DIPHTHERIAL PHAGES

Last year we reported studies on the properties of various lysogenic diphtheria bacilli and of the phages liberated from them following induction by ultraviolet light. The isolation of a fast-growing defective lysogenic, toxigenic strain P(βd) was described and its properties were compared with other normal lysogenic strains, P(β) and C7(β) and with the respiratory-defective high toxin-producing strain PW8. These studies have been continued during the past year and some progress has been made towards finding out the nature of the defects in phages derived from P(βd) and PW8.

Preparation of P32-labelled phage: The Casamino acid medium that we use in the laboratory for growth of C. diphtheriae contains about 600-640mμ inorganic phosphorus (Pi) per liter, derived mainly from Casamino acids. It is necessary to remove most of this phosphorus in order to prepare P32-phage of high specific activity. Casamino acids were treated with a slight excess of $CaCl_2.2H_2O$ at pH 8.5 in the presence of 7μc P132 at 100C. The precipitated calcium phosphate was removed by filtration leaving only 20-40mg Pi per liter. The filtrate was brought to pH7 and the excess Ca was then removed by treatment with Dowex Chelating Resin A-1. The medium was then supplemented with Mueller's solution II containing Mg, vitamins, and trace elements and with 1.5% maltose.

For preparation of P32-labelled phage, cultures of P(β) were grown overnight in the low Pi medium and then inoculated to OD590 = 0.15 in low Pi medium to which 10μc/ml carrier-free P32 had been added. After about 2 hours at 37C when the OD reached 0.5 (4 x 10^8 bacteria/ml), the culture was irradiated with UV. Two hours later, 1% normal rabbit serum and M/50 sodium citrate was added. The burst was complete 3 hours after irradiation. After centrifugation, the clear supernate was passed through a Millipore #65 filter and then through a Sephadex G50 column equilibrated with 1% Casamino acids at pH 7.8 containing 5×10^{-5}M chloramphenicol. The final yield of β-phage averaged about 5×10^9 plaque-forming particles per ml. The preparation contained ca 20,000 cpm/ml P32 of which about 5% was incorporated in phage. About double this specific activity was obtained by using G200 instead of G50 Sephadex. P32-labelled lysates from P(β), P(βd) and PW8 were prepared by the above procedure.

I^{131}-labelled anti-phage rabbit gammaglobulin: A potent antiserum prepared by immunizing rabbits with high titer Bv phage was exhaustively absorbed with suspensions of the sensitive non-toxigenic C7(-) strain. The absorbed serum, specific for phage head proteins, was dialysed against 0.01M phosphate pH7, passed through a DEAE column and then eluted with 0.02M phosphate. The eluate was precipitated with an equal volume of saturated ammonium sulfate. The precipitate was redissolved and passed through Sephadex equilibrated with Tris at pH 8.

Fourteen ml of the globulin fraction containing a total of 100 mg protein was trace-labelled with 2mc I^{131} (ca 3 atoms per molecule globulin) according to the method of Cohen et al (8).

When first prepared, the fraction contained 1.8×10^7 cpm/ml or 1.8×10^6 cpm/mg protein of β-radiation. The radioactivity was about 5% specifically precipitable by purified Bc phage.

Reaction of I^{131}-antiphage globulin with suspensions of diphtheria bacilli: Suspensions of C7(-) bacteria are not agglutinated by the absorbed antiphage globulin. Nevertheless, even after exhaustive absorption with the C7 strain, I^{131}-labelled globulin continues to be taken up by the bacteria and cannot be removed by washing the treated bacteria on Millipore filters. That the reaction is not an antigen-antibody interaction is proved by the fact that the I^{131} uptake is independent of the number of bacteria or the density of the suspension and is directly proportional to the concentration but not the amount of I^{131}-globulin used in the test. Moreover, the amount of label taken up is markedly less when the C7(-) suspensions are treated with antiphage in the presence of 5% bovine gammaglobulin.

Reaction of I^{131}-antiphage with phage-sensitized bacteria: When 4 or more phage particles per bacterium are absorbed to suspensions of the C7 strain, the organisms become agglutinable by antiphage serum. The agglutinates may be centrifuged, suspended in medium containing 0.5% BGG, collected on Millipore filters and counted for I^{131}. In antibody excess, the I^{131} count is directly proportional to the phage taken up by the bacteria, up to a multiplicity of nearly 300, provided that the I^{131} count of similarly treated unsensitized bacteria is subtracted. This blank is relatively small when the reaction is carried out in a medium containing 5mg/ml BGG.

The β-phages have unusually long tails (2400 Å) and after absorption to bacteria and treatment with antiphage become extremely sensitive to breakage by shear. A single vigorous washing in a pipet, is sufficient to break off the phage particles and reduce the absorbed I^{131} counts to the level of the control suspension. This fact has proved useful in double label experiments designed to demonstrate absorption followed by penetration of P32-labelled phage DNA.

When suspensions of purified β from UV-lysates of P(β) are absorbed to C7, the organisms become agglutinable by antiphage serum and take up an amount of I^{131} equivalent to the phage titer. Moreover, the defective, non-plaque-forming βd mutant likewise renders C7 suspensions agglutinable and from the I^{131} uptake it can be calculated that the burst size of P(βd) is approximately the same as that of the normal P(β) strain. Finally, using βd labelled with P32, it has been shown that the βd DNA is injected into the C7 cell and remains there after the phage coat has been broken off. Thus despite the fact that βd produces no plaques on C7 and that thus far we have been unable to convert C7 to C7(βd), the defective phage adsorbs to C7 normally and injects its DNA normally. In the electron microscope, βd appears to be indistinguishable from β.

The behavior of the classic PH8 strain is quite different.

UV-lysates of PW8, prepared in exactly the same way as those from P(β) and P(βd), fail to render C7 suspensions agglutinable by antiphage serum and the treated bacteria take up no measurable I^{131}. It has been shown using PW8 UV-lysates concentrated 15-20 fold, that a few normal-appearing phage particles can be seen in electron micrographs. However, the present experiments with I^{131}-antiphage, show that less than 2% as many particles are liberated from PW8 as from an equivalent number of irradiated P(β) or P(βd) organisms. PW8 lysates labelled with P32, contain a small amount of labelled material that adsorbs to C7 and cannot be removed by washing. It seems unlikely that this material is phage DNA. It may possibly be labelled polyphosphate which is known to be present in diphtheria bacilli.

REFERENCES

1. Strauss, N. & Hendee, E. D., J. Exp. Med. 112 351 (1960)
2. Lennox, E. S. & Kaplan, A. S., Proc. Soc. Exp. Biol. Med. 95 700 (1957)
3. Gabliks, J. and Solotorofsky, M., J. Immunol. 88 505 (1962)
4. Yoneda, M., Brit. J. Exp. Path.
5. Allen, E. H. & Schweet, R. S., J. Biol. Chem. 237 760 (1962)
6. Dintzis, H. M., Proc. Nat. Acad. Science 47 247 (1961)
7. Warner, J. R., Knopf, P.M. & Rich, A. Proc. Nat. Acad. Sc. 49122 (1963)
8. Cohen, S., Holloway, R. C., Matthews, C., & McFarlane, A.S., Biochem. J. 62 144 (1956).